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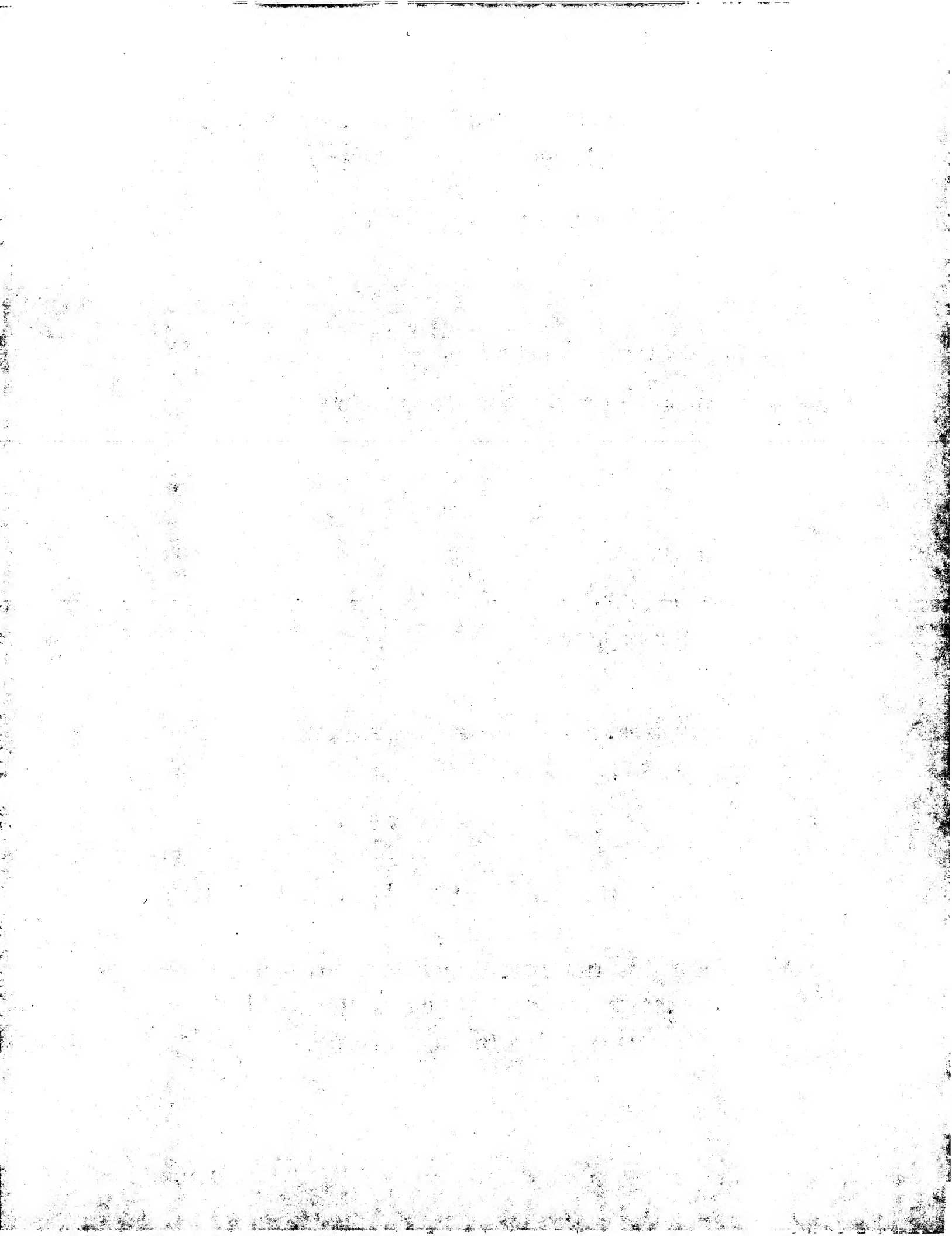
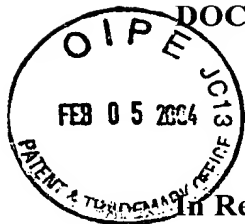


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AF / QP 1632

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PATENT



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: Antoniou and Grosveld

Confirmation No. 7091

Serial No.: 09/247,054

Group Art Unit: 1632

Filing Date: February 9, 1999

Examiner: Anne Marie Falk

For: **SELF-REPLICATING EPISOMAL EXPRESSION VECTORS CONFERRING
TISSUE-SPECIFIC GENE EXPRESSION**

EXPRESS MAIL LABEL NO. EL964 551 822US
DATE OF DEPOSIT: February 5, 2004

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P.O. Box 1450
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TRANSMITTAL LETTER FOR CERTIFIED COPY OF PRIORITY DOCUMENT

Attached please find the certified copy of the foreign application from which priority is claimed for this case:

Country: Great Britain
Application No.: GB 9617214.3
Filing Date: 16 August 1996

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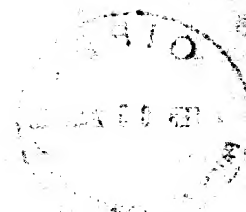
Respectfully submitted,

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Dated: February 5, 2004

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VECTOR

The invention relates to stable self-replicating episomal
5 expression vectors for expressing a gene of interest in a
host cell.

Background of the Invention

10 The expression of a foreign gene in a host cell is generally
achieved by transferring the gene into the host cell using
a gene transfer vector. Gene transfer vectors are available
in the art and include for example retrovirus vectors,
adenoviral vectors and adenoassociated viral vectors. Many
15 transfer vectors operate by integrating at least the
transferred gene, if not the complete gene transfer vector,
into the host cell chromosome, although non-integrating
transfer vectors are known in the art. The efficiency of
stable integration of transfected gene constructs is
20 generally very inefficient ($1: 10^3 - 10^6$). The disadvantage
of using these viral based gene transfer vectors is that the
amount of genetic material that the vector is able to
accommodate is limited by the genetic packaging limitations
of the virus. Gene transfer vectors which include large
25 fragments of inserted genetic material are difficult to
produce at a sufficiently high titre to be of practical
value. Therefore, it is difficult to include additional
genetic material in a virus-based vector, for example, gene
regulatory elements, without deleteriously affecting stable
30 gene transfer.

Locus Control Regions (LCRs) (Grosveld et al., *Cell* 51:975-
985 (1987)), also known as Dominant Activator Sequences,
Locus Activating Regions or Dominant Control Regions, confer
35 tissue-specific, integration-site independent, copy number-
dependent expression on a linked gene that has been
integrated into the chromosome of a host cell. LCRs were
originally discovered in the human globin gene system, which

exhibited strong position effects when integrated into a chromosome of a host cell in a tissue of a transgenic mouse or a mouse erythro-leukaemia (MEL) cell (see, for example, Magram et al., *Nature* 315:338-340 (1985); Townes et al., *EMBO J.* 4:1715-1723 (1985); Kollias et al., *Cell* 46:89-94(1986); Antoniou et al., *EMBO J.* 7:377-384 (1988)). Position effects were overcome when a LCR was linked directly to such transgenes (Grosveld et al., supra). Other LCRs have since been identified, including the CD2 LCR which promotes gene expression in T cells (see, for example, Greaves et al., *Cell* 56:979 (1989), European Patent Application EP-A-0 668 357), the macrophage-specific lysozyme LCR (Bonifer et al., *EMBO J.* 9:2843-2848 (1990), Bonifer et al., *NAR* 22:4202-4210 (1994)), and a class II MHC LCR (Carson and Wiles, *Nucleic Acids Res.* 21,9:2065-2072 (1993)).

It is an object of the invention to provide a stable gene transfer system which, when present in a host cell, confers stable and tissue-restricted expression of a gene of interest carried in a self-replicating episomal vector.

Summary of the Invention

This invention provides self-replicating, LCR-containing, episomal expression vectors into which a gene of interest is inserted for expression of the gene in cells of a specific tissue-type.

The invention therefore encompasses a self-replicating episomal DNA expression vector for expressing a gene of interest in a host cell in a tissue-restricted manner, the vector comprising: (a) an origin of replication capable of directing replication of the DNA expression vector in cells of the specific type of tissue; and (b) an LCR, or component thereof, which when operatively linked to a gene of interest and present in a host cell directs expression of the gene in a tissue-restricted manner.

A vector according to the invention also may include the gene of interest inserted into a cloning site and operatively linked to the LCR.

5

The term "gene" is used to define any DNA sequence capable of being expressed. The gene of interest may be a foreign or heterologous gene, that is, a gene that is either not normally found in the genomic DNA of the host cell or is not normally expressed in that host cell. The gene of interest
10 may also be an artificial DNA sequence.

The tissue-restricted expression of a gene of interest is mediated in vectors of the invention using an appropriate
15 LCR, or components or portions thereof, which confer tissue-type specific expression on the gene of interest.

As used herein, a "locus control region" (LCR) is defined as a genetic element which is obtained from a tissue-specific locus of a eukaryotic host cell and which, when
20 linked to a gene of interest and integrated into a chromosome of a host cell, confers tissue-specific, integration-site independent (position independent), copy number-dependent expression on the gene of interest. An LCR that is useful according to the invention possesses these
25 characteristics when integrated into chromosomal DNA, and will retain the ability to confer tissue-type restricted expression of a linked gene when present in a self-replicating episomal vector according to the invention. A
30 "component" of an LCR refers to a portion of an LCR that also confers tissue-restricted gene expression when linked to a gene and integrated into a self-replicating episomal vector. An LCR may be identified structurally in that it is associated with one or more DNase I hypersensitive sites
35 in its natural chromosomal context, and a component of an LCR useful according to the invention will also encompass at least one DNase I hypersensitive site.

Preferably, the self-replicating episomal DNA expression vector of the present invention comprises a component of an LCR, wherein preferably the component of an LCR is a DNA fragment of the human β -globin LCR encompassing HS2, HS3 and HS4. It is further preferred that the component of an LCR encompasses HS2, HS3 and HS4 only.

It is further preferred that the component of an LCR is a DNA fragment of the human β -globin LCR encompassing HS3. It is further preferred that the component of an LCR encompasses HS3 only.

The hypersensitive sites of the human β -globin LCR are described by Tuan et al. (PNAS USA, 82, 6384-6388, (1985)).

15

The invention also encompasses a pair of vectors comprising a self-replicating episomal DNA expression system for expressing a gene of interest in a host cell in a tissue-restricted manner, the pair of vectors comprising:

20 a first vector comprising:

- (a) an origin of replication;
- (b) an LCR, or component thereof, which when operatively linked to a gene of interest and present in a host cell directs expression of the gene in a tissue-restricted manner; and
- (c) a cloning site for a gene of interest; and

25

a second vector comprising:

- (a) an origin of replication; and
- (b) a sequence encoding a replication factor, the replication factor being necessary for replication of the origin of replication.

30

In another embodiment, the second vector also may include an LCR or component thereof, which may be the same LCR or component thereof as is present on the first vector or may be a different LCR or component thereof which specifies the same or at least an overlapping tissue specificity as the first LCR or component thereof such that the gene of

35

interest and the replication sequence are expressed in some of the same cells.

It is preferred that for red cell-restricted gene expression, the β -globin LCR, preferably from the human β -globin locus, is used. As used herein, "red cells" refer to cells of erythroid lineage. It is preferred that for T-cell restricted gene expression, the CD2 LCR from the CD2 locus be used. It is preferred that for class II MHC-bearing cell restricted gene expression the class II MHC LCR be used. It also is preferred that for macrophage cell restricted gene expression the lysozyme LCR be used.

The term "episomal vector" refers to a nucleic acid vector which may be linear or circular, and which is usually double-stranded in form. A vector according to the invention is generally within the size range of 1kb - 1,000kb, the preferred size range being on the order of 5kb - 100kb.

20

The terms "self-replicating", "stably maintained" and "persistence" are defined herein as follows. The self-replicating function of a vector of the invention enables the vectors to be stably maintained in cells, independently of genomic DNA replication, and to persist in progeny cells for three or more cell divisions without a significant loss in copy number of the vector in the cells, i.e., without loss of greater than an average of about 50% of the vector molecules in progeny cells between a given cell division. This self-replicating function may be provided by using a viral origin of replication and optionally providing one or more viral replication factor that may be required for replication mediated by that particular viral origin. Origins of replication and, if necessary, any replication factor may be used from a variety of viruses, including Epstein-Barr virus (EBV), human and bovine papilloma viruses, and papovavirus BK. The self-replicating function may alternatively be provided by one or more mammalian

sequences such as described by Wohlgemuth et al (Gene Therapy, 3, 503-512, (1996)), Vos et al (J. Cell. Biol., Supplement 21A, 433, (1995)) and Sun et al (Nature Genetics, 8, 33-41, (1994)), optionally in combination with one or
5 more sequences which may be required for nuclear retention. The advantage of using mammalian, especially human sequences for providing the self-replicating function is that no extraneous activation factors etc. are required which could have toxic or oncogenic properties.

10

In a preferred embodiment, the origin of replication is a viral origin of replication. Preferably, the viral origin of replication is the oriP of EBV and the replication factor is the trans-acting EBNA-1 protein. EBNA-1 may be provided
15 by expression of the EBNA-1 gene on the same episomal expression vector carrying OriP or on another vector in the cell or from an EBNA-1 gene in the genomic DNA of the host cell.

20 In a preferred embodiment, a gene encoding a replication factor may be present on the self-replicating episomal vector, i.e., on either the same vector that carries the gene of interest or on another vector of a pair of vectors, and operatively linked to an LCR.

25

As used herein, "linked" refers to a cis-linkage in which the gene of interest and/or the gene encoding an replication factor and the LCR are contained in the same vector and thus present in cis on the same DNA, and "operatively linked"
30 refers to a cis linkage in which the gene of interest and/or replication gene is subject to tissue-restricted expression via the LCR.

Optionally, a transcription terminator may be introduced
35 into a vector of the invention, preferably between the LCR, or component thereof, and the promoter of the gene of interest or the gene(s) encoding replication factor(s) to prevent undesirable transcription of these genes from other

promoters that may be present on the vectors. Alternatively, a transcription terminator may be introduced upstream of the LCR and downstream of the gene of interest or replication gene(s).

5

The episomal expression vectors of the invention may be delivered to cells *in vivo*, *ex vivo*, or *in vitro* by any of a variety of the methods employed to deliver DNA molecules to cells. The vectors may also be delivered alone or in the
10 form of a pharmaceutical composition that enhances delivery to cells in the body.

In a preferred embodiment, the vectors of this invention are used in gene therapy to express a therapeutically useful
15 protein in the cells of a specific diseased tissue, including tumor tissue, in the body.

Vectors of the invention also are used to express a therapeutically useful protein in cells of a specific
20 tissue-type *in vitro*.

A further embodiment of the present invention is the self-replicating episomal vector of the present invention for use in therapy.

25

A further embodiment of the present invention is the use of the self-replicating episomal vector in the manufacture of a composition for the treatment of a disease. Preferably, the disease is a disease treatable by gene therapy including
30 proliferative diseases and viral infections. The composition may also be used in vaccination treatments and in prodrug therapies.

In another embodiment of this invention, the vectors
35 described herein are used to produce a type of transgenic animal in which a foreign or heterologous gene is expressed only in a specific tissue type, as directed by the LCR, or component thereof, incorporated into the vector. The self-

replicating function of the vector ensures that the vector will be passed on to the progeny of the transgenic animal. Such transgenic animals are particularly useful in testing the fidelity and efficacy of tissue-specific gene expression prior to clinical treatment of humans.

Brief Description of the Drawings

Fig. 1 shows a schematic map of an EBV based self-replicating expression vector p220.2. p220.2 is a 8952 bp plasmid which encodes EBNA-1, OriP and hygromycin resistance. It replicates as plasmid in 143 and HeLa cells. EBNA-1 in this construct is driven off an unknown promoter located in the pBR322 sequences. DNA inserted upstream of EBNA-1 appears to eliminate expression of EBNA-1. bp 1-35 are pBR322. bp 36-2646 are EBV EBNA-1 107567-110176 (Baer et al., *Nature*, 310, 207-211, 1984) BamHI-PvuII fragment. The BamHI site was blunt-end ligated to the HindIII site. bp 2647-4826 are EBV OriP 7333-9516 SphI-SstII sites blunt-end ligated to the BstEII site (Sugden et al., *MCB*, 5, 410-413, 1985). bp 4827-5460 are HSV TK regulatory region (McKnight, *NAR*, 8, 5949-5964, 1980). bp 6488-6747 are a HSV TK PvuII fragment ligated into poisonless pBR322 at NaeI site. This site is lost in cloning. bp 5461-6487 are the HPH gene (Gritz and Davies, *Gene*, 25, 179-188, 1983) BamHI fragment blunt end ligated into the SmaI and BglII sites in HSV TK sequences. bp 6748-8952 are pBR322 poisonless vector (deletion of 1.1 kb in pBR322) confers ampicillin resistance (Lusky & Botchan, *Nature*, 293, 79-81, 1981). The polylinker form pUC 12 (SmaI-HaeIII fragment) is inserted into a NarI site within the HSV TK sequences. (//) denotes blunt-end ligations.

Fig. 2 shows a reporter gene construct:

a) the β -globin gene extending from a 5' Hpa I site at -815 bp to an EcoRV site 1685 bp past the poly A addition site in the plasmid GSE1758 (Talbot et al., *EMBO J.*, 9, 2169-2178, 1990) was removed as a 4.1 kb EcoRV fragment and

inserted into a blunted SalI site in the polylinker of p220.2 (Fig. 1). This cloning step brings a number of extra restriction sites (including a unique SalI site) 5' of the β -globin gene.

5

Fig. 3 shows β -Globin LCR Hypersensitive site constructs. Constructs which contain more than one hypersensitive site were designed such that the site order matches that of the wild type β -globin locus. SalI linkers were added to both
 10 the 5' and 3' ends allowing the DNA to be cloned into the unique SalI site upstream of the β -globin gene in the p220.2 reporter vector (Fig. 2).

Fig. 4 shows:

15 A) An autoradiogram of an S1 nuclease analysis of RNA from K562 cells transfected with self-replicating, episomal, expression vectors containing a human β -globin reporter gene operatively linked to various combinations of HS components from the β -globin LCR. Lane β 1 (β -globin gene alone), lanes
 20 2β 1 and 2 (HS2 operatively linked to β -globin gene), lanes 3β 1 and 2 (HS3 operatively linked to β -globin gene), lanes 4β 1 and 2 (HS4 operatively linked to β -globin gene), lanes 4β 1 and 2 (HS4 operatively linked to β -globin gene), lanes 23β 1 and 2 (HS2/HS3 combination operatively linked to β -
 25 globin gene), lanes 34β 1 and 2 (HS3/HS4 combination operatively linked to β -globin gene), lanes 24β 1 and 2 (HS2/HS4 combination operatively linked to β -globin gene), lanes 234β 1 and 2 (HS2/HS3/HS4 combination operatively linked to β -globin gene). Lane +Gamma refers to
 30 untranslated K562 cells as a negative control, and lane +beta refers to MEL cells transfected with a β -globin gene plus β LCR combination which acts as a positive control. All lanes are independent transfections.

35 B) End labelled DNA probes from the 5'-region of the β -globin gene is shown schematically including the S1 protected fragments.

Detailed Description of the Invention

The contents of all references referred to herein are
5 incorporated by reference thereto.

The invention is based on the discovery that a locus control
region or component thereof may be used in a stable, self-
replicating episomal vector containing a gene of interest to
10 specify tissue-restricted expression of the gene of
interest.

The stable maintenance of an episomal vector containing an
LCR in a host cell is achieved by using an origin of
15 replication that is operative to initiate and replicate an
episomal DNA independent of the host cell chromosomes and if
necessary an origin replication factor.

Origins of Replication and Replication Factors Useful 20 According to the Invention

Plasmid vectors for expressing heterologous genes in
eukaryotic cells have been made using a portion of the
genomic DNA of one or more eukaryotic viruses such as
25 Epstein Barr Virus, human papova virus BK, adenovirus-based
vectors, and bovine and human papilloma viruses, or from
sequences derived from mammalian genomic DNA including human
genomic DNA (see Wohlgemuth et al (1996)).

30 Episomal vectors of the invention comprise a portion of a
genomic DNA that encodes an origin of replication (ori) or
a sequence capable of providing efficient autonomous
replication, which is required for such vectors to be self-
replicating and, thus, to persist in a host cell over
35 several generations. In addition, an episomal vector of the
invention also may contain one or more genes encoding
proteins that are required for replication, i.e.,
replicator protein(s). Optionally, the replicator

protein(s) which help initiate replication may be expressed in trans on another DNA molecule, such as on another vector or on the host genomic DNA, in the host cell containing a self-replicating episomal expression vector of this invention.

Preferred self-replicating episomal LCR-containing expression vectors of the invention do not contain viral sequences that are not required for long-term stable maintenance in a eukaryotic host cell such as regions of a viral genome DNA encoding core or capsid proteins that would produce infectious viral particles or viral oncogenic sequences which may be present in the full-length viral genomic DNA molecule.

The term "stable maintenance" herein, refers to the ability of a self-replicating episomal expression vector of this invention to persist or be maintained in non-dividing cells or in progeny cells of dividing cells in the absence of continuous selection without a significant loss (e.g., >50%) in copy number of the vector for two and preferably five or more generations. The most preferred vectors will be maintained over 10-15 or more cell generations. In contrast, "transient" or "short-term" persistence of a plasmid in a host cell refers to the inability of a vector to replicate and segregate in a host cell in a stable manner; that is, the vector will be lost after one or two generations, or will undergo a loss of >51% of its copy number between successive generations.

Several representative self-replicating, LCR-containing, episomal vectors of this invention are described further below. It will be understood by one of skill in the art that the invention is not limited to any one origin of replication or any one episomal vector, but encompasses the combination of the tissue-restricted control of an LCR in an episomal vector.

1. Epstein-Barr Virus-Based Self-Replicating Episomal Expression Vectors Useful According to the Invention.

The latent origin oriP from Epstein-Barr Virus (EBV) is
5 described in (Yates et al., *Proc. Natl. Acad. Sci USA*
81:3806-3810 (1984); Yates et al., *Nature* 313:812-815
(1985); Krysan et al., *Mol. Cell. Biol*, 9:1026-1033 (1989);
James et al., *Gene* 86: 233-239 (1990), Peterson and
Legerski, *Gene* 107:279-284 (1991); and Pan et al., *Som. Cell*
10 *Molec. Genet.* 18:163-177 (1992)). An EBV-based episomal
vector useful according to the invention will contain the
oriP region of EBV which is carried on a 2.61 kb fragment of
EBV (see Fig. 1) and the EBNA-1 gene which is carried on a
2.18 kb fragment of EBV (see Fig. 1). For a general
15 discussion of EBV-based episomal vectors see Yates J. L., et
al., *Nature*, 313, 812-815, (1985). One vector which carries
the oriP and EBNA-1 gene of EBV is shown in Fig. 1. The
vector shown in Fig. 1 also contains an antibiotic
resistance gene for selection of stable transfected
20 eukaryotic cells in culture, a polylinker cloning site for
insertion of a gene of interest, and a portion of pBR322 for
production of vector DNA in bacterial host cells.

The EBNA-1 protein, which is the only viral gene product
25 required to support *in trans* episomal replication of vectors
containing oriP, may be provided on the same episomal
expression vector containing oriP (see, for example, James
et al., *supra*; Peterson and Legerski, *supra*; Pan et al.,
supra). It is also understood, that as with any protein
30 such as EBNA-1 known to be required to support replication
of viral plasmid *in trans*, the gene also may be expressed on
another DNA molecule, such as a different DNA vector or the
host genomic DNA, in the host cell containing the episomal
expression vector of the invention.

2. Papilloma Virus-Based, Self-Replicating, Episomal Expression Vectors Useful According to the Invention.

5 The episomal expression vectors of the invention also may be based on replication functions of the papilloma family of virus, including but not limited to Bovine Papilloma Virus (BPV) and Human Papilloma Viruses (HPVs). BPV and HPVs persist as stably maintained plasmids in mammalian cells. 10 trans-acting factors encoded by BPV and HPVs, namely E1 and E2, have also been identified which are necessary and sufficient to mediate replication in many cell types via a minimal origin of replication (Ustav et al., *EMBO J.* 10: 449-457 (1991); Ustav et al., *EMBO J* 10:4231-4329, (1991); 15 Ustav et al., *Proc. Natl. Acad. Sci. USA* 90: 898-902 (1993)).

An episomal vector useful according to the invention is the BPV-I vector system described in Piirsoo et al., *EMBO J.*, 20 15:1 (1996) and in WO 94/12629. The BPV-1 vector system described in Piirsoo et al. comprises a plasmid harbouring the BPV-1 origin of replication (minimal origin plus extrachromosomal maintenance element) and optionally the E1 and E2 genes. The BPV-1 E1 and E2 genes are required for 25 stable maintenance of a BPV episomal vector. These factors ensure that the plasmid is replicated to a stable copy number of up to thirty copies per cell independent of cell cycle status. The gene construct therefore persists stably in both dividing and non-dividing cells. This allows the 30 maintenance of the gene construct in cells such as hemopoietic stem cells and more committed precursor cells.

The BPV origin of replication has been located at the 3' end of the upstream regulatory region within a 60 base pair 35 (bp) DNA fragment (nucleotides (nt) 7914 - 7927) which includes binding sites for the E1 and E2 replication factors. The minimal origin of replication of HPV has also been characterized and located in the URR fragment (nt 7022-

7927) of HPV (see, for example, Chiang et al., *Proc. Natl. Acad. Sci. USA*, 89:5799-5803 (1992)).

As used herein, "E1" refers to the protein encoded by
 5 nucleotides (nt) 849-2663 of BPV subtype 1 or by nt 832-2779
 of HPV of subtype 11, to equivalent E1 proteins of other
 papilloma viruses, or to functional fragments or mutants of
 a papilloma virus E1 protein, i.e., fragments or mutants of
 E1 which possess the replicating properties of E1.

10

As used herein, "E2" refers to the protein encoded by nt
 2594-3837 of BPV subtype 1 or by nt 2723-3823 of HPV subtype
 11, to equivalent E2 proteins of other papilloma viruses, or
 to functional fragments or mutants of a papilloma virus E2
 15 protein, i.e., fragments or mutants of E2 which possess the
 replicating properties of E2.

"Minichromosomal maintenance element" (MME) refers to the
 extrachromosomal maintenance element of the papilloma viral
 20 genome to which viral or human proteins essential for
 papilloma viral replication bind, which region is essential
 for stable episomal maintenance of the papilloma viral MO in
 a host cell, as described in Piirsoo et al. (supra).
 Preferably, the MME is a sequence containing multiple
 25 binding sites for the transcriptional activator E2. The MME
 in BPV is herein defined as the region of BPV located within
 the upstream regulatory region which includes a minimum of
 about six sequential E2 binding sites, and which gives
 optimum stable maintenance with about ten sequential E2
 30 binding sites. E2 binding site 9 is a preferred sequence
 for this site, as described hereinbelow, wherein the
 sequential sites are separated by a spacer of about 4-10
 nucleotides, and optimally 6 nucleotides. E1 and E2 can be
 provided to the plasmid either in cis or in trans, also as
 35 described in WO 94/12629 and in Piirsoo et al. (supra).

"E2 binding site" refers to the minimum sequence of
 papillomavirus double-stranded DNA to which the E2 protein

binds. An E2 binding site may include the sequence 5' ACCGTTGCCGGT 3', which is high affinity E2 binding site 9 of the BPV-1 URR; alternatively, an E2 binding site may include permutations of binding site 9, which permutations are found
5 within the URR, and fall within the generic E2 binding sequence 5' ACCN6GGT 3', where N6 represents 6 nucleotides (N), and where N is, independently in each of the 6 positions, any nucleotide. One or more transcriptional activator E2 binding sites are, in most papillomaviruses,
10 located in the upstream regulatory region, as in BPV and HPV.

A vector which also is useful according to the invention may include a region of BPV between 6959 - 7945/1 - 470 on
15 the BPV genetic map (as described in WO 94/12629), which region includes an origin of replication, a first promoter operatively associated with a gene of interest, the BPV E1 gene operatively associated with a second promoter to drive transcription of the E1 gene; and the BPV E2 gene
20 operatively associated with a third promoter to drive transcription of the E2 gene.

E1 and E2 from BPV will replicate vectors containing the BPV origin or the origin of many HPV subtypes (Chiang et al
25 supra). E1 and E2 from HPV will replicate vectors via the BPV origin and via the origin of many HPV subtypes (Chiang et al., supra). As with all vectors of the invention, the BPV-based episomal expression vectors of the invention must persist through 2-5 or more divisions of the host cell.

30

3. Papovavirus-Based, Self-Replicating, Episomal Expression Vectors Useful According to the Invention.

The vectors of the invention also may be derived from a
35 human papovavirus BK genomic DNA molecule. For example, the BK viral genome can be digested with restriction enzymes EcoRI and BamHI to produce a 5 kilobase (kb) fragment that contains the BK viral origin of replication sequences that

can confer stable maintenance on vectors (see, for example, De Benedetti and Rhoads, *Nucleic Acids Res.* 19:1925 (1991), as can a 3.2 kb fragment of the BK virus (Cooper and Miron, *Human Gene Therapy* 4:557 (1993)).

5

4. Regulation of Origin of Replication Factor Expression.

As noted above, the vectors of the invention may contain one or more genes encoding a trans-acting viral replication factor required for stable maintenance of the vectors in host cells.

The regulatory elements i.e. promoters, enhancers, LCRs etc., which drive expression of the replicator gene(s) may be identical or different to the regulatory elements which drive expression of the gene of interest, and may provide identical or overlapping tissue-specific expression of the replicator gene and the gene of interest. For example, for expression in B-cells, the immunoglobulin heavy chain promoter/enhancer or the Ig heavy or light chain promoters can be used. For tumors that are present in an unspecified cell type, a promoter from a ubiquitously expressed gene, for example from the phosphoglycerolkinase, IE-CMV, RSV-LTR or DHFR genes may be used. The arrangement of replicator gene(s) relative to the episomal vector origin of replication may mimic the natural orientations of these sequences in the genome, or it may assume a variety of other orientations, the choices of which will be apparent to one of skill in the art.

30

In EBV-based episomal vectors, it is often preferable that the transcription of a viral gene encoding a replication factor be placed under the control of a relatively weak promoter. For example, expression of the gene encoding EBNA-1 can be toxic to most cells and/or result in undesirable rearrangements in vector sequences when the gene is expressed using the CMV promoter, a well known, relatively strong, eukaryotic promoter. In contrast,

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expression of the EBNA-1 gene using a relatively weak eukaryotic promoter sequence permits EBNA-1 to function to maintain a desirable level (i.e., a level that is non-toxic to cells in culture) of vector replication in host cells.

- 5 Such relatively weak promoter sequences useful in the vectors of the invention are found in a portion of the sequence of the bacterial plasmid pBR322 and the thymidine kinase (tk) promoter.
- 10 Preferred promoters for E1 and E2 expression in papillomavirus-based vectors include the thymidine kinase promoter, the SV40 early promoter, the CMV promoter and the SR α promoter.
- 15 5. Manufacture of Vector DNA.

In another embodiment of the invention, vectors are made comprising culturing a cell containing the vector of the present invention in order to produce sufficient DNA for use
20 according to the invention. It is particularly preferred that such manufacture occurs in lower eukaryotic cells, e.g., yeast or insect, or prokaryotic cells, e.g., bacterial cells such as *E. coli* or *Salmonella*. Therefore, it is preferred that a vector of this invention will further
25 comprise an origin of replication of yeast, insect or bacterial origin, e.g., the pBR322 origin of replication, and one or more genes encoding a selectable marker, e.g., a gene encoding kanamycin resistance, for selection of cells containing the vector and/or a marker gene such as Lac Z.

30

LCRs, and Components Thereof, For Providing Tissue-Specific Gene Expression Useful According to the Invention

The invention contemplates the combination of a stable,
35 self-replicating episomal vector and a locus control region or component thereof for tissue-type restricted expression of a gene contained in the vector.

Without being bound to any one theory, it is believed that, in the genomic DNA context, the LCR promotes an opening of the usually tightly wound structure of the chromatin in cells of a specific tissue type and thereby promotes transcription of the genes in the region of the opened chromatin in a tissue-specific manner.

In addition, LCRs are capable of conferring tissue-restricted gene expression whether they are located upstream or downstream of a linked gene, and regardless of orientation with respect to the gene. LCRs useful in an episomal vector according to the invention may be placed upstream or downstream or in any orientation with respect to the linked gene.

Prior to the invention, it was not known whether an LCR, when present in a self replicating episomal rather than a chromosomal context, could confer tissue-restricted gene expression. Furthermore, in transient transfections of a vector, LCR components were found not to increase expression levels unless the LCR component comprised a classical enhancer element (Tuan et al., PNAS USA 86, 2544-2558 (1989)).

In the self-replicating, episomal expression vectors described herein, an LCR, or component thereof, is operatively linked to a selected gene present on the vectors such that the gene expression occurs only in cells of the particular tissue type in which the LCR, or component thereof, is known to function. While not wishing to be limited by a mechanism, it would appear that the self-replicating vectors described herein are stably maintained in a conformation similar to that of the host cell's natural chromatin which the LCR, or component thereof, may be capable of opening and/or then promoting the transcription of sequences to which the LCR, or component thereof, is linked.

In general, whether an LCR or component, or combination of components thereof can be used to mediate tissue specific expression of a foreign gene is easily assayed using a self-replicating episomal vector containing the LCR or component thereof operatively linked to a reporter gene. Expression of the reporter gene in a tissue-specific manner can be assayed using standard methods and by comparing reporter gene expression in cells of the tissue type for which the LCR is specific versus reporter gene expression in cells of a different tissue type. A variety of reporter genes and vectors containing reporter genes are commercially available which can be used for testing promoter and/or enhancer functions. Standard reporter genes used in the art include the lacZ gene encoding β -galactosidase, the CAT gene encoding chloramphenicol acetyl transferase, the luciferase gene system, and the gene encoding alkaline phosphatase (see, for example, James et al., (supra); Peterson and Legerski, (supra); Pan et al., (supra)).

For example, the β -globin LCR contains well-defined portions which are DNA fragments encompassing DNase I hypersensitive sites, i.e., HS1, HS2, HS3, and HS4, located upstream of the embryonic ϵ globin gene in the β -globin locus (Tuan et al., 1985). It has been discovered that, when a combination of HS2, HS3, and HS4 components is used in an episomal vector according to the invention, the combination of LCR components is highly effective for obtaining tissue-specific expression of a foreign gene in erythroid cells. It also has been discovered that the HS3 component alone is effective in conferring tissue specific expression of a linked gene as demonstrated in the Examples below; however, the HS2 component, which is known to possess classical enhancer activity (Tuan et al., (1989), PNAS, 86, 2554-2558), does not possess this activity when present as the sole LCR component on the episomal vector.

Additionally LCRs useful according to the invention include but are not limited to the CD2 LCR which promotes gene

expression in T cells (see, for example, Greaves et al., (supra)) the macrophage-specific lysozyme LCR (Bonifer et al., (supra)), and a class II MHC LCR (Carson et al., (supra)).

5

Optionally, one or more transcription terminators may also be incorporated into the vectors of the invention to prevent undesirable transcription from other known or latent promoter sequences in the vector which might proceed through (i.e., transcriptional readthrough) the gene whose expression has been placed under the exclusive control of a particular LCR, or component thereof. An example of such a useful transcription terminator is the β -globin terminator, though other eukaryotic transcription terminators also may be used in the vectors described herein. Preferably, the transcription terminator is placed between the LCR, or component thereof, and the promoter of the gene to which the LCR is operatively linked.

20 Selecting Heterologous or Foreign Genes of Interest

The vectors of the invention are particularly useful in expressing nucleic acid sequences such as heterologous or foreign genes, or fragments thereof in cells of a specific tissue-type.

Examples of therapeutic nucleic acid sequences which may be incorporated into an episomal vector include the following. Therapeutically useful nucleic acid sequences include sequences encoding receptors, enzymes, ligands, regulatory factors, and structural proteins. Therapeutic nucleic acid sequences also include sequences encoding nuclear proteins, cytoplasmic proteins, mitochondrial proteins, secreted proteins, plasmalemma-associated proteins, serum proteins, viral antigens, bacterial antigens, protozoal antigens and parasitic antigens. Nucleic acid sequences useful according to the invention also include sequences encoding proteins, lipoproteins, glycoproteins, phosphoproteins and nucleic

acid (e.g., RNAs or antisense nucleic acids). Proteins or polypeptides which can be expressed using the episomal vector of the present invention include hormones, growth factors, enzymes, clotting factors, apolipoproteins, 5 receptors, drugs, oncogenes, tumor antigens, tumor suppressors, viral antigens, parasitic antigens, bacterial antigens and prodrug activating polypeptides. Specific examples of these compounds include proinsulin, growth hormone, androgen receptors, insulin-like growth factor I, 10 insulin-like growth factor II, insulin-like growth factor binding proteins, epidermal growth factor TGF- α , TGF- β , PDGF, angiogenesis factors (acidic fibroblast growth factor, basic fibroblast growth factor and angiogenin), matrix proteins (Type IV collagen, Type VII collagen, laminin), 15 phenylalanine hydroxylase, tyrosine hydroxylase, oncogenes (ras, fos, myc, erb, src, sis, jun), E6 or E7 transforming sequence, p53 protein, Rb gene product, cytokine receptor, IL-1, IL-6, IL-8, viral capsid protein, and proteins from viral, bacterial and parasitic organisms which can be used 20 to induce an immunologic response, prodrug activating polypeptides including plasmin, carboxypeptidaseG2, thymidine kinase (viral), cytosine deaminase, glucose oxidase, xanthine oxidase, carboxypeptidase A, α -galactosidase, β -glucosidase, azoreductase, γ -glutamyl 25 transferase, β -glucuronidase, β -lactamase, alkaline phosphatase, penicillin amidase or cytochrome P-450, DT diaphorase and nitroreductases, and other proteins of useful significance in the body. The nucleic acid sequence which can be incorporated into the episomal vector may be 30 artificial sequences or modified naturally occurring sequences. The nucleic acid sequence which can be incorporated is only limited by the availability of the nucleic acid sequence encoding the protein or polypeptide to be incorporated. One skilled in the art will readily 35 recognize that as more proteins and polypeptides become identified their coding sequences can be integrated into the episomal vector and expressed in the animal or human tissue.

Vectors of the invention also may be used to express genes that are already expressed in a host cell (i.e., a native or homologous gene), for example, to increase the dosage of the gene product. It should be noted, however, that expression of a homologous gene may result in deregulated expression which may not be subject to control by the LCR, or component thereof, due to its over-expression in the cell.

Vector Delivery

10

Numerous techniques are known and useful according to the invention for delivering self-replicating, LCR (or component thereof)-containing, episomal expression vectors described herein cells including the use of nucleic acid condensing agents, electroporation, complexation with asbestos, polybrene, DEAE cellulose, Dextran, liposomes, lipopolyamines, polyornithine, particle bombardment and direct microinjection (reviewed by Kucherlapati and Skoultchi, *Crit. Rev. Biochem.* 16:349-379 (1984); Keown et al., *Methods Enzymol.* 185:527 (1990)).

20

A vector of the invention may be delivered to a host cell non-specifically or specifically (i.e., to a designated subset of host cells) via a viral or non-viral means of delivery. Preferred delivery methods of viral origin include viral particle-producing packaging cell lines as transfection recipients for the vector of the present invention into which viral packaging signals have been engineered, such as those of adenovirus, herpes viruses and papovaviruses. Preferred non-viral based gene delivery means and methods may also be used in the invention and include nucleic acid condensing peptides, encapsulation in liposomes, and transfection of cells *ex vivo* with subsequent reimplantation or administration of the transfected cells.

35

Various peptides derived from the amino acid sequences of viral envelope proteins have been used in gene transfer when co-administered with polylysine DNA complexes (Plank et al.,

J. Biol. Chem. 269:12918-12924 (1994));. Trubetskoy et al., *Bioconjugate Chem.* 3:323-327 (1992); WO 91/17773; WO 92/19287; and Mack et al., *Am. J. Med. Sci.* 307:138-143 (1994)) suggest that co-condensation of polylysine
 5 conjugates with cationic lipids can lead to improvement in gene transfer efficiency. PCT publication number WO 95/02698 discloses the use of viral components to attempt to increase the efficiency of cationic lipid gene transfer.

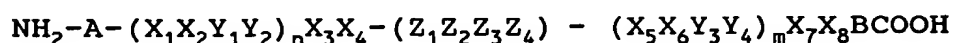
- 10 Nucleic acid condensing agents useful in the invention include spermine, spermine derivatives, histones, cationic peptides and polylysine. Spermine derivatives refers to analogues and derivatives of spermine and include compounds as set forth in International Publication No. WO 93/18759
 15 (published September 30, 1993).

Disulfide bonds have been used to link the peptidic components of a delivery vehicle (Cotten et al., *Meth. Enzymol.* 217:618-644 (1992)); see also, Trubetskoy et al.
 20 (supra).

Delivery vehicles for delivery of DNA constructs to cells are known in the art and include DNA/poly-cation complexes which are specific for a cell surface receptor, as described
 25 in, for example, Wu and Wu, *J. Biol. Chem.* 263:14621 (1988); Wilson et al., *J. Biol. Chem.* 267:963-967 (1992); and U.S. Patent No. 5,166,320).

Delivery of a vector according to the invention is
 30 contemplated using nucleic acid condensing peptides. A nucleic acid condensing peptide which is particularly useful for condensing the nucleic acid construct and therefore for delivering nucleic acid to a cell includes an amino acid sequence of the generic formula

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wherein each of X₁₋₈ is, independently, an amino acid having

a positively charged group on the side chain; wherein each of Y_{1-4} is, independently, a naturally occurring amino acid which promotes alpha helix formation; wherein each of Z_{1-4} is, independently, a naturally occurring amino acid with at least 3 amino acids having a high propensity to form a stabilized turn structure; wherein A is an amino-terminal serine or threonine residue; wherein B is any amino acid; and wherein $n = 2 - 4$ and $m = 2$.

Other peptides are those wherein each of X_{1-8} is, independently, lysine, arginine, 2.4-diamino-butyric acid or ornithine; wherein each of Y_{1-4} is, independently, glutamic acid, alanine, leucine, methionine, glutamine, tryptophan or histidine; wherein each of Z_{1-4} is, independently, asparagine, glycine, proline, serine, and aspartic acid; wherein B is any one of alanine, glutamic acid or cysteine.

It is also contemplated according to the invention that peptides useful in this embodiment of the invention which involves delivery of a nucleic acid to a cell either *ex vivo* or *in vivo* may contain one or more internal Serine, Threonine, or Cysteine residues, preferably at a position in the sequence which will be exposed for conjugation to a selected ligand, and thus not on the positively charged (nucleic acid oriented) face of the α -helix. This positioning of selected reactive amino acid residues within the peptide are oriented such that they do not contact the face of the peptide that contacts nucleic acid permits conjugation of the peptide with other functional peptides by bonds of selected and defined stability. Cysteine allows specific conjugation via the thiol side chain to compounds containing other reactive thiol groups (via disulfides), alkylating functions (to form thioether bonds), or other thiol reactive groups such as maleimide derivatives.

35

Peptides which fall within this generic sequence include:

NBC7 NH_2 -TRRAWRRRAKRRRAARRCGVSARRAARRAWRRE-COOH; and

NBC11 NH_2 -TKKAWKKAEKKAAKCGVSAKKAACKKAWKKA- CONH_2 .

Thus, a nucleic acid condensing peptide useful for delivery of a nucleic acid may contain: 1) helix-forming amino acids,
 5 2) a repeating three-dimensional structure that contacts the major groove of the nucleic acid, 3) suitable chromophores for quantitation, and 4) a number of "handles" (i.e., reactive sites) for regio-specific conjugation of ligands which form accessory functional domains.

10

Nucleic acid condensing peptides also may include portions of H1 (sequence I, II or III below) or sequences from other human histones (sequence IV below) which are identified herein as sequences which possess the ability to condense
 15 nucleic acid. Therefore, a nucleic acid condensing peptide can include a linear combination of the following three consensus sequences where the total sequence length is >17 residues:

20 Sequence I: -K-K-X-P-K-K-Y-Z-B-P-A-J-

where: K is Lysine, P is Proline; A is Alanine; X is Serine, Threonine or Proline; Y is Alanine or Valine; Z is Alanine, Threonine or Proline; B is Lysine, Alanine, Threonine or
 25 Valine; and J is Alanine or Valine.

Sequence II: -X-K-S-P-A-K-A-K-A-

where: X is Alanine or Valine; K is Lysine; S is Serine; P
 30 is Proline; and A is Alanine.

Sequence III: -X-Y-V-K-P-K-A-A-K-Z-K-B-

where: X is Lysine or Arginine; Y is Alanine or Threonine;
 35 Z is Proline, Alanine or Serine; B is Lysine, Threonine or Valine; K is Lysine; P is Proline; A is Alanine.

Sequence IV

-A-B-C-D-E-F-G-H-I-J-K-

where: A is preferably Lysine or Threonine; B is preferably
 5 Glycine or Glutamine; C is preferably Glycine, but can also
 be Aspartate, Glutamate, or Serine; D is preferably Glycine,
 but can also be Lysine, Valine, Glutamine, or Threonine; E
 is preferably Lysine or Alanine; F is preferably Alanine or
 Lysine, G is preferably Arginine, but can also be Valine or
 10 Isoleucine; H is preferably Alanine, but can also be
 Threonine, Histidine, or Proline; I is preferably Lysine,
 Arginine, or Glutamine; J is Alanine or Anginine; and K is
 preferably Lysine or Glutamine. A preferred consensus
 sequence is:

15

-K-G-G-G-K-A-R-A-K-A-K-.

One such peptide is NBC1, which has the following
 structure:

20

NH_2 -[SV40 NLS]-[Seq I]-[Seq II]-[Seq III]-[SV40 NLS]-[Seq
 I]-C-COOH, where -C- is Cysteine; where the SV40 NLS has the
 sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Gln; and the sequence

25 NH_2 -PKKKRKVEKKSPKKAKKPAKSPAKAKAKAVKPKAAKPKKPKKKRKVEKKSP
 KKAKKPAAC (Acm)-COOH.

Another such nucleic acid condensing peptide of the
 invention will have an amino acid sequence that falls within
 30 the following generic sequence:

NH_2 -X-(Y)_n-C-COOH, where X is either absent or Serine or
 Threonine; Y is sequence I, II or III as defined above; n is
 2-6; and C is Cysteine.

35

Other such peptides have the following structures and
 sequences: NBC2 has the structure: NH_2 -[Seq III]-[SV40
 NLS1]-[Seq I]-C-COOH, where -C- is Cysteine.

NBC8 has the structure: $\text{NH}_2\text{--[Seq I]--[Seq I]-C-COOH}$, where -C- is Cysteine.

- 5 NBC9 has the structure: $\text{NH}_2\text{--[Seq I]--[Seq I]--[Seq I]-C-COOH}$, where -C- is Cysteine.

NBC10 has the structure: $\text{NH}_2\text{--[Seq I]--[Seq I]--[Seq I]--[Seq I]-C-COOH}$

- 10 where -C- is Cysteine; the amino acid sequences of which are as follows:

NBC2 $\text{NH}_2\text{-KPAAKPKKPKKKRKVEKKSPKKAKKPAAC(Acm)-COOH}$;

NBC8 $\text{NH}_2\text{-KKSPKKAKKPAAKKSPKKAKKPAYC(Acm)-COOH}$;

- 15 NBC9 $\text{NH}_2\text{-TKKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAC(Acm)-COOH}$;

NBC10 $\text{NH}_2\text{-KKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAAKKSPKKAKKPAYC(Acm)-COOH}$.

- As described above, nucleic acid condensing peptides having
20 a low polydispersion index (PDI) are useful for delivery to a cell of a nucleic acid according to the invention. The PDI for such peptides may be calculated from analysis of the peptides by electro-spray mass spectrometry. This method gives the exact mass of each component to within
25 0.001%. The PDI values of the peptide preparations useful in the present invention are in the range of 1.0 - 1.100. Peptide preparations which are especially useful in the invention possess a PDI <1.01, and even <1.001.

- 30 The first nucleic acid condensing peptide may include 8-24 positively charged amino acid side groups; for example, the number of positively charged amino acid side groups may be in the range of 12-18.

- 35 The ratio of positive/negative charges in a synthetic virus like particle that is capable of targeting a specific mammalian cell type is within the range 0.5-3 per phosphate residue in the nucleic acid; this ratio thus also may be

within the range 0.8 - 1.2.

The ratio of positive/negative charges in a synthetic virus like particle that is unrestricted with respect to the type
5 of cell it targets is in within the range of 0.5 - 5 per phosphate residue in the nucleic acid, and thus also may be within the range of 1.2 - 2.

Functional groups may be bound to peptides useful for
10 delivery of a vector according to the invention. These functional groups may include a ligand that targets a specific cell-type such as a monoclonal antibody, insulin, transferrin, asialoglycoprotein, or a sugar. The ligand
15 thus may target cells in a non-specific manner or in a specific manner that is restricted with respect to cell type.

The functional group also may comprise a lipid, such as palmitoyl, oleyl, or stearoyl; a neutral hydrophilic polymer
20 such as polyethylene glycol (PEG), or polyvinylpyrrolidone (PVP); a fusogenic peptide such as the HA peptide of influenza virus; or a recombinase or an integrase. The functional group also may comprise an intracellular trafficking protein such as a nuclear localization sequence
25 (NLS).

In a particularly preferred embodiment for delivery, that is, wherein the second functional group is covalently linked to a first functional group which is linked directly to the
30 nucleic acid condensing peptide, the first functional group may comprise one of a lipid or a neutral hydrophilic polymer such as PEG and the second functional group may comprise a ligand that targets a cell surface receptor. For example, when the first functional group comprises a lipid, the
35 second functional group may comprise a ligand that targets a cellular receptor. When the first functional group comprises PEG, the second functional group may comprise a ligand that targets a cellular receptor. The ligand may be,

for example, one of a sugar moiety or a ligand whose cellular receptor is restricted to a cell-type, and thus the target cell population may be unrestricted or restricted as to cell type. Alternatively, when the first functional group comprises a lipid, the second functional group may comprise PEG.

The above-described nucleic acid condensing peptide/vector DNA composition may be prepared as follows. The composition is formulated such that the nucleic acid and the peptide preparation are prepared in equal volumes of the same buffer (usually 0.15M to 1.0M NaCl; 25mM HEPES, pH 7.4). The nucleic acid is shaken or vortexed while the condensing peptide preparation is added at the rate of 0.1 volume per minute. The complex is left at room temperature for at least 30 minutes prior to addition to the target cells or prior to administration to a subject, and can be stored at 4°C. The particle is centrifuged to remove any aggregated material.

In addition to the above-described DNA/polycation complexes for cell targeting, methods are known in the prior art for preparing cell-targeting liposomes containing nucleic acid. Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in size from 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver nucleic acids to cells and that the nucleic acid remains biologically active.

Furthermore, a cationic liposome delivery vehicle originally designed as a research tool, Lipofectin, has been shown to deliver intact mRNA molecules to cells yielding production of the corresponding protein. In this situation, the DNA to be delivered is absorbed on to the surface of the liposome.

Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display long circulation

half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system. An example of targeting liposomes is immunoliposomes. Liposomes are prepared, for example, by adsorption of proteins (e.g., immunoglobulin) on the liposomal surface; incorporation of native protein into the liposome membrane during its formation (e.g., by ultrasonication, detergent dialysis or reverse phase evaporation); covalent binding (direct or via a spacer group) of a protein to reactive compounds incorporated into the liposomes membrane; noncovalent hydrophobic binding of modified proteins during liposome formation or by the incubation with preformed liposomes); and indirect binding, including covalent binding of immunoglobulin protein via a polymer to the liposome (see Torchilin, V.P. *CRC Critical Reviews in Therapeutic Drug Carrier Systems*, 2(1)). Binding of the nucleic acid-ligand complex to the receptor facilitates uptake of the nucleic acid by receptor-mediated endocytosis.

A nucleic acid-ligand complex linked to adenovirus capsids, which naturally disrupt endosomes, thereby releasing material into the cytoplasm, can be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel et al., *Proc. Natl. Acad. Sci. USA* 88:8850 (1991); Cristiano et al., *Proc. Natl. Acad. Sci USA* 90:2122-2126 (1993)).

Receptor-mediated nucleic acid uptake can be used to introduce nucleic acid into cells either *in vitro* or *in vivo* and, additionally, has the added feature that nucleic acid can be selectively targeted to a particular cell type by use of a ligand which binds to a receptor selectively expressed on a target cell of interest, or can be non-selective with respect to the target cell type.

The precise stoichiometric ratio of the various components of the delivery vehicle can be varied in order to control the magnitude of the initial immune response, the efficiency of delivery and the degree of specific targeting to cells.

In the case of non-specific delivery to cells, a non-specific ligand may be used that targets a cell surface receptor; in the case of specific delivery, a ligand may be used that targets a specific subset of cells. For example, soluble DNA/polylysine complexes can be generated (Li et al., *Biochem. J.* 12:1763 (1973)). Polylysine complexes tagged with asialoglycoprotein have been used to target DNA to hepatocytes *in vitro* (Wu and Wu, *J. Biol. Chem.* 262:4429 (1987); U.S. Patent 5,166,320). Lactosylated polylysine (Midoux et al., *Nucleic Acids Res.* 21:871-878 (1993)) and galactosylated histones (Chen et al., *Human Gene Therapy* 5:429-435 (1994)) have been used to target plasmid DNA to cells bearing lectin receptors, and insulin conjugated to polylysine (Rosenkrantz et al., *Exp. Cell Res.* 199:323-329 (1992)) to cells bearing insulin receptors. Monoclonal antibodies have been used to target DNA to particular cell types (Machy et al., *Proc. Natl. Acad. Sci. USA* 85:8027-8031 (1988); Trubetskov et al., *supra* and WO 91/17773 and WO 92/19287).

In a preferred embodiment, a ligand is included in the delivery vehicle for targeting a vector of the invention to cells of a specific type of tissue. Preferably, the ligand is specific for an epitope expressed on the surface of cells of a particular type of tissue. For certain cancer tissue, preferred ligands include antibodies or fragments thereof, such as monoclonal antibody C242 which recognizes the CA242 domain of CanAg expressed on the colon cancers (Lindholm et al., *Int. Arch. Allergy Appl. Immunol.* 71:171-181 (1983) and Larson et al., *Int. J. Cancer* 42:877-882 (1983)); monoclonal antibody SM3, which recognizes polymorphic epithelial mucin (PEM) expressed by breast cancer cells (Burchell et al.,

Cancer Research 47:5476-5482 (1983)) and monoclonal antibodies recognizing a novel epitope found on the epidermal growth factor receptor of human glial tumors but not on normal tissues (Moscatello et al., *Cancer Res.* 55:5536-5539 (1995)). Furthermore, growth factors can also be used to target tumors. For example, the epidermal growth factor (EGF) receptor is over expressed on lung cancer cells and thus EGF can be used as the ligand to target the delivery vehicle to lung cancers (Cristiano et al., *Cancer Gene Therapy* 3:4-10 (1996)).

In addition, certain soluble macromolecules can be used for passive tumor targeting of certain tumor types. Many solid tumors possess vasculature that is hyperpermeable to macromolecules. Although the reasons for this are not clearly understood, the result is that such tumors can selectively accumulate circulating macromolecules. The enhanced permeability and retention effect (EPR effect) is thought to constitute the mechanism of action of SMANCS (styrene/maleic-anhydride-neocarzinostatin), now in regular clinical use in Japan for the treatment of hepatoma. Another class of conjugates under investigation for anticancer activity is N-(2-hydroxypropyl) methacrylamide copolymer-anthracycline conjugates (Seymour, L., *Critical Reviews in Therapeutic Drug Carrier Systems* 9(2):135-187 (1992)). Thus, a polymer comprising styrene/maleic-anhydride or N-(2-hydroxy-propyl)methacrylamide copolymer can be used as a ligand to target the delivery vehicle of the present invention.

Pharmaceutical Compositions and Therapeutic Use

The pharmaceutical compositions of the present invention may comprise a self-replicating episomal vector or delivery composition of the present invention, if desired, in admixture with a pharmaceutically acceptable carrier or diluent, for therapy to treat a disease or provide the cells of a particular tissue with an advantageous protein or

function.

A self-replicating episomal, LCR-containing vector of the invention or composition or delivery vehicle comprising a
5 vector of the invention may be administered via a route which includes intramuscular, intravenous, aerosol, oral (tablet or pill form), topical, systemic, ocular, as a suppository, intraperitoneal and/or intrathecal and direct injection of the vector DNA or delivery vehicle into a tumor
10 mass.

The exact dosage regime will, of course, need to be determined by individual clinicians for individual patients and this, in turn, will be controlled by the exact nature of
15 the protein expressed by the gene of interest, and the type of tissue that is being targeted for treatment.

The dosage also will depend upon the disease indication and the route of administration. Advantageously, the vectors of
20 the invention are designed to provide a long-term expression of a selected gene only in the cells of a specific tissue, i.e., in the host cells of tissue in which the LCR, or component thereof, is functional. Thus, in such host cells, duration of treatment will generally be continuous or until
25 the cells die. The number of doses will depend upon the disease, and efficacy data from clinical trials.

The amount of vector DNA delivered for effective gene therapy according to the invention will be in the range of
30 between about 50 ng -1000 μ g of vector DNA/kg body weight; and preferably in the range of between about 1-100 μ g vector DNA/kg.

Although it is preferred according to the invention to
35 administer the episomal vector to a mammal for *in vivo* cell uptake, a vector as described herein may be administered utilizing an *ex vivo* approach whereby cells are removed from an animal, transduced with the episomal vector containing a

selected gene under the control of a particular LCR, or component thereof, and then re-implanted into the animal. The liver, for example, can be accessed by an *ex vivo* approach by removing hepatocytes from an animal, transducing
 5 the hepatocytes *in vitro* with the episomal vector containing a gene encoding a selected protein or therapeutic nucleic acid, and re-implanting the transduced hepatocytes into the animal (e.g., as described for rabbits by Chowdhury et al., *Science* 254:1802-1805, 1991, or in humans by Wilson, *Hum.*
 10 *Gene Ther.* 3:179-222, 1992). Such methods also may be effective for delivering an expression vector of the invention to various populations of cells in the circulatory or lymphatic systems, such as erythrocytes, T cells, and B cells.

15

Transgenic Animals For Testing Tissue Specificity or Efficacy of Gene Therapy

In another embodiment of the invention, there is provided
 20 a mammalian model for determining the tissue-specificity and/or efficacy of gene therapy using a self-replicating episomal LCR-containing vector of the invention. The mammalian model comprises a transgenic animal whose cells contain the vector of the present invention. Methods of
 25 making transgenic mice (Gordon et al., *Proc. Natl. Acad. Sci. USA* 77:7380 (1980); Harbers et al., *Nature* 293:540 (1981); Wagner et al., *Proc. Natl. Acad. Sci. USA* 78:5016 (1981); and Wagner et al., *Proc. Natl. Acad. Sci. USA* 78:6376 (1981), sheep, pigs, chickens (see Hammer et al.,
 30 *Nature* 315:680 (1985)), etc., are well-known in the art and are contemplated for use according to the invention. It should be noted that EBV based vectors do not work in rodents but only in primates and humans. However, BPV based vectors do work in rodents. Such animals permit testing
 35 prior to clinical trials in humans.

For example, the transgenic animal may have a tumor or the propensity to develop a tumor. Stewart et al., *Int. J.*

Cancer 53:1023 (1993)) describe a transgenic mouse having a T-cell tumor. Teitz et al., *Proc. Natl. Acad. Sci. USA* 90:2910 (1993)) describe transgenic mice having rhabdomyosarcomas and insulin-producing pancreatic-islet tumors. An episomal vector containing a selected gene to be expressed in the tumor cells alone may be transduced into ova from any of these tumor-bearing mice to create an animal model for determining whether the LCR, or component thereof, strictly limits expression of the selected gene to tumor cells and also to determine the result of expressing the selected gene in such tumor cells.

Transgenic animals containing self-replicating, LCR-containing episomal vectors of the invention also may be used for long-term production of a protein of interest in a tissue-specific manner; for example, for production of human globin in red cells of the transgenic animal using the β -globin LCR or the HS 2,3, and 4 combination or HS3 alone, or production of a protein of interest in macrophages of a transgenic animal using the LCR macrophage/lysozyme LCR.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner. The preparation, testing and analysis of several representative constructs of the invention is described in detail below. One of skill in the art may adapt these procedures for preparation and testing of other vectors of the invention using different episomal vectors, different LCRs, or components thereof, and different genes of interest.

EXAMPLE 1

Preparation of Test Gene Constructs and Vector Systems

1. EBV Vector

One EBV-based vector useful according to the invention is the p220.2 vector (Fig. 1). The p220.2 vector contains (i)

the OriP and EBNA-1 gene from EBV, (ii) a hygromycin resistance gene for selection of stable transfected eukaryotic tissue culture cells, (iii) a polylinker cloning site for test genes and (iv) a pBR322 vector backbone which
 5 contains a weak eukaryotic promoter sequence which drives expression of the EBNA-1 gene in eukaryotic cells.

2. Reporter Gene

10 The reporter gene (Fig. 2) used to assess LCR activity was derived from human β -globin: 4.1 kb HpaI-EcoRV fragment with 815 base pairs (bp) 5' and 1685 bp 3' flanking sequences (Fritsch et al., *Cell* 19:959-972 (1980); Lawn et al., *Cell* 19:959-972 (1980))

15

The reporter gene was cloned by blunt-end ligation into the unique polylinker HindIII site of p220.2 (Fig. 2).

3. β -globin LCR Test Constructs

20

Three β -globin LCR components are the DNase 1 hypersensitive sites HS2, HS3 and HS4 (Collis et al., *EMBO J.* 9:223-240 (1990)). These components were cloned as 1.5 - 2 kb fragments individually or in combinations of two or three
 25 sites (see below) upstream of the β -globin reporter gene in the unique SalI site of p220.2 (see, Fig. 3).

EXAMPLE 2

Analysis of Stable Transfection of Tissue Culture Cells

30

EBV-based vectors are most stable as self-replicating episomes in primate or human cells. Therefore stable transfections of the β -globin LCR test gene constructs were carried out in the human myelogenous leukemia cell line K562
 35 (Lozzio and Lozzio, *Blood* 45:321-334 (1975)). This cell line displays an embryonic (ϵ) and foetal (γ) pattern of globin gene expression (Anderson et al., *Int. J. Cancer* 23:143-147 (1979)) and has previously been successfully used

to assess β -globin LCR function (Blom van Assendelft et al., Cell 56:969-977 (1989)).

1. Transfection Method

5

K562 cells (10^7) were transfected by electroporation with 50 μ g of supercoiled test construct DNA using a Bio Rad Gene Pulser (BioRad, Hercules, CA) set at 960 μ F and 300 V as described in Antoniou, 1991. Each electroporated sample was
10 divided equally between two 75 cm² tissue culture flasks in order to generate two independent pools of stable transfected cells. Hygromycin B (250 μ g/ml) was added 24 hours after electroporation and maintained at this concentration thereafter. Cell death of non-transfected
15 cells was evident after 7-10 days (equivalent to about 10 cell divisions) and confluent flasks of transfected cells were obtained 7 days later.

2. RNA and DNA Preparation from Transfected Cells

20

(i) Total RNA was prepared by selective precipitation in the presence of 3 M LiCl and 6 M urea (Auffrey and Rougeon, Eur. J. Biochem 107:303-314 (1980)).

25 (ii) Total genomic DNA was prepared from isolated nuclei. Cells ($\sim 2 \times 10^7$) were disrupted in RSB (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl₂) plus 0.1% NP40 followed by centrifugation to pellet the nuclei. The nuclei were subsequently lysed by resuspending in TNE (150mM Tris-HCl
30 (pH 7.5), 100mM NaCl, 5mM EDTA), 1% SDS and 50 μ g/ml proteinase K and incubated overnight at 55°C. This was followed by one extraction with an equal volume of phenol/chloroform (1:1) and the DNA precipitated from the aqueous phase with 0.7 volumes of isopropanol. The
35 resulting DNA precipitate was spooled out of the solution, washed once in 70% ethanol and dissolved in 200 μ l of TE (10 mM Tris-Cl. 0.1 mM EDTA, pH 8.0).

3. Analysis for Expression of Test Genes

Transfected gene expression was assessed by an S1-nuclease protection assay (Antoniou et al., *Human Genetic Disease*
5 *Analysis. A Practical Approach* (second edition) (1993) using an end labeled DNA probe from the 5'- region of the β -globin (Antoniou et al., (1988). The probe detects only those transcripts which have arisen by initiation from the correct, wild-type cap site (Fig. 4). As an internal
10 standard, all samples were simultaneously assayed for the endogenous γ -globin mRNA using a probe from the 3'-end of the gene (Fig. 4, lower panel).

4. Assessing Episomal Status and Copy Number of Transfected 15 DNA

(a) Episomal Status.

Total DNA (10 μ g) from isolated nuclei was double digested
20 with PvuI and DpnI (6 units) overnight and separated by electrophoresis on a 0.6% agarose gel and Southern blotted onto Magnacharge Plus nylon transfer membranes. Digestion with DpnI ensures any residual bacterially derived plasmid DNA is destroyed. The blot was then probed with the p220.2
25 vector labeled with 32 P by nick-translation. Transfected DNA in the form of episomes appear as linear molecules of 12-17.5 kb depending on the size of the test construct.

(b) Episome Copy Number

30

The same quantity of DNA was digested with EcoRI and DpnI, and Southern blotted as above. The blot was probed with an 920 bp BamHI-EcoRI fragment spanning intron II of the β -globin gene. This results in the detection of a 5.5 kb
35 fragment from the three copies of the endogenous β -globin gene and 7.2 - 12.7kb fragment from the transfected constructs.

EXAMPLE 3

1. Expression Analysis of β -Globin Reported Gene Constructs

5 Figure 4 (upper panel) shows the autoradiogram from the analysis of RNA by an S1-nuclease protection assay for the expression of the transfected gene constructs. Quantitation was by Phosphorimager (Molecular Dynamics) and is summarized in Table 1.

10

Little or no expression was seen with the β -globin gene alone (no LCR component present in the self-replicating episomal vector) (lane $\beta 1$). A vector containing either HS2 or HS4 did not improve expression to a significant extent
15 (lanes 2β and 4β). In contrast, a vector containing HS3 (lanes 3β) alone gave expression levels that were at least 10 times greater than a vector containing either HS2 or HS4 alone. The level of expression obtained with a vector containing HS3 was generally better than any of the
20 constructs with two HS sites, combinations of HS2/HS3 (lanes 23β) or a vector containing a combination of HS3/HS4 (lanes 34β). The vector containing a combination of all three β -globin LCR elements (HS2,3,4) (lanes 234β) gave expression that was slightly higher (upto 2 fold) than HS3 alone or the
25 HS2/HS3 and HS3/HS4 two-site constructs.

The results obtained using a self-replicating β -globin-LCR or LCR-component-containing episomal vector containing the β -globin gene as a reporter gene demonstrate that the β -
30 globin LCR HS2/,3/4, HS2/3, HS3/4, HS2/4 or HS3 alone are effective for conferring tissue-specific gene expression on an episomal vector.

Of the single β -globin LCR HS sites tested, only a vector
35 containing HS3 gives significant levels of gene expression, with vectors containing HS2 or HS4 being at least an order of magnitude less effective. The construct containing all three HS sites functioned most efficiently. Therefore, a

classical enhancer, such as HS2 (localized to a NF-E2/AP-1 dimer binding site within it's core), (Tuan et. al., *Proc. Natl. Acad. Sci. USA* 86:2554-2558 (1989); Ney et. al., *Genes Dev.* 4:993-1006 (1990a); and Ney et al., *Nuc. Acids Res.* 18:6011-6017 (1990b) is distinguishable from an LCR or LCR component specifying tissue-specificity by the inability of the former to confer a useful level of transcription according to the invention.

Construct	Endogenous/ Exogenous $\times 10^2$	Endogenous γ signal	Exogenous β signal
HS2,3,4 β -2	32	856.338	273.22
15 HS2,3,4 β -1	28	581.853	163.35
HS3 β -1	27	309.087	84.487
HS3 β -2	19	899.079	168.217
HS3,2 β -2	16	342.098	55.253
HS4,2 β -2	15	2407.205	360.629
20 HS4,2 β -1	14	2737.317	377.922
HS3,2 β -1	12	1181.171	143.282
HS4,3 β -2	9	720.397	66.132
HS4,3 β -1	9	604.471	54.556
β -1	7	1385.387	93.697
25 HS2 β -1	6	433.451	25.151
HS2 β -2	6	421.97	24.077
HS4 β -1	5	502.573	25.731

Table 1: Expression of the human β -globin under the control of the β LCR in self-replicating EBV-based episomal vectors in K562 cells.

This table shows the quantitation of the S1 nuclease protection assay (Figure 4) of RNA from K562 cells stably transfected with either the human β -globin gene alone (β -1) or under the control of different combinations of the DNaseI hypersensitive (HS) sites of the β LCR in the self-replicating EBV-based vector p220.2 (Figure 3) using a

Molecular Dynamics phosphorimager. The data show that high levels of β -globin expression can be obtained with HS3 (HS3 β -1/2) which possesses an authentic chromatin opening capability but not from HS2 (HS2 β -1/2) which contains only
5 classical enhancer activity or HS4 (HS4 β -1). Two HS site combinations (HS2,3; HS3,4; HS2,4) are no better than HS3 alone. The three site combination (HS2,3,4) gives the highest level of expression.

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CLAIMS

1. A self-replicating episomal DNA expression vector for
expressing a gene of interest in a host cell in a tissue-
5 restricted manner, the vector comprising:

(a) a self-replicating origin of replication; and
(b) an LCR, or component thereof, which when operatively
linked to a gene of interest and present in a host cell
10 directs expression of said gene in a tissue-restricted
manner.

2. The self-replicating episomal DNA expression vector
of claim 1, further comprising a gene of interest
15 operatively linked to the LCR, or component thereof.

3. The self-replicating episomal DNA expression vector
of claim 2 wherein the component of an LCR is a component of
the β -globin LCR which encompasses H2, HS3 and HS4.
20

4. The self-replicating episomal DNA expression vector
of claim 3 wherein the component of an LCR is a component of
the β -globin LCR which encompasses HS3 only.

25 5. The self-replicating episomal DNA vector of any one
of the previous claims, said origin of replication being a
viral origin of replication.

6. The self-replicating episomal DNA expression vector
30 of claim 5 wherein the viral origin of replication is an
origin of replication from a virus selected from the group
consisting of Epstein-Barr virus, papilloma virus, and
papovavirus BK.

35 7. The self-replicating episomal DNA expression vector
of claims 1 to 6 further comprising a sequence encoding a
replication factor required for replication of the
expression vector in a host cell.

8. The self-replicating episomal DNA expression vector of claim 7 wherein the sequence encoding the replication factor is selected from the group consisting of a sequence
5 encoding EBNA-1 of Epstein-Barr virus, a sequence encoding E1 of papilloma virus, and a sequence encoding E2 of papilloma virus.

9. The self-replicating episomal DNA expression vector
10 of any one of the previous claims, further comprising an antibiotic resistance gene for selecting cells in culture stably transfected with the expression vector.

10. The self-replicating episomal DNA expression vector
15 of any one of the previous claims, further comprising a eukaryotic transcription termination sequence placed between the LCR and the gene of interest and operative to prevent transcription therebetween.

20 11. A pair of vectors comprising a self-replicating episomal DNA expression system for expressing a gene of interest in a host cell in a tissue-restricted manner, the pair of vectors comprising:

- i. a first vector comprising:
25 (a) an origin of replication;
(b) an LCR, or component thereof, which when operatively linked to a gene of interest and present in a host cell directs expression of said gene in a tissue-restricted manner; and
30 (c) a cloning site for a gene of interest; and
- ii. a second vector comprising:
(a) an origin of replication; and
(b) a sequence encoding a replication factor, said replication factor being necessary for replication
35 of said origin of replication.

12. The pair of vectors of claim 11, further comprising a gene of interest operatively linked to the LCR, or

component thereof.

13. The pair of vectors of claim 11 or claim 12 wherein the origin of replication is a viral origin of replication.

5

14. The pair of vectors of claim 13, said viral origin of replication being from a virus selected from the group consisting of Epstein-Barr virus, papilloma virus, and papovavirus BK.

10

15. The pair of vectors of any one of claims 11 to 14 wherein the sequence encoding the replication factor is selected from the group consisting of a sequence encoding EBNA-1 of Epstein-Barr virus, a sequence encoding E1 of papilloma virus, and a sequence encoding E2 of papilloma virus.

16. The pair of vectors of any one of claims 11 to 15, each said first and second vector further comprising, individually, an antibiotic resistance gene for selecting cells in culture stably transfected with the expression vector.

17. The pair of vectors of any one of claims 11 to 16, said first vector further comprising a eukaryotic transcription termination sequence placed between the LCR and the cloning site for the gene of interest.

18. A method for expressing a gene of interest in cells of a specific tissue-type comprising administering a self-replicating episomal DNA expression vector of any one of claims 2 to 10 or a pair of vectors of any one of claims 12 to 17 to a mammal.

19. A method of obtaining persistent, tissue-specific expression of a gene of interest in a host cell in culture, comprising culturing a host cell transfected with the vector of any one of claims 2 to 10 or the pair of vectors of any

one of claims 12 to 17.

20. The vector of any one of claims 2 to 10 or the pair of vectors of any one of claims 12 to 17 for use in therapy.

5

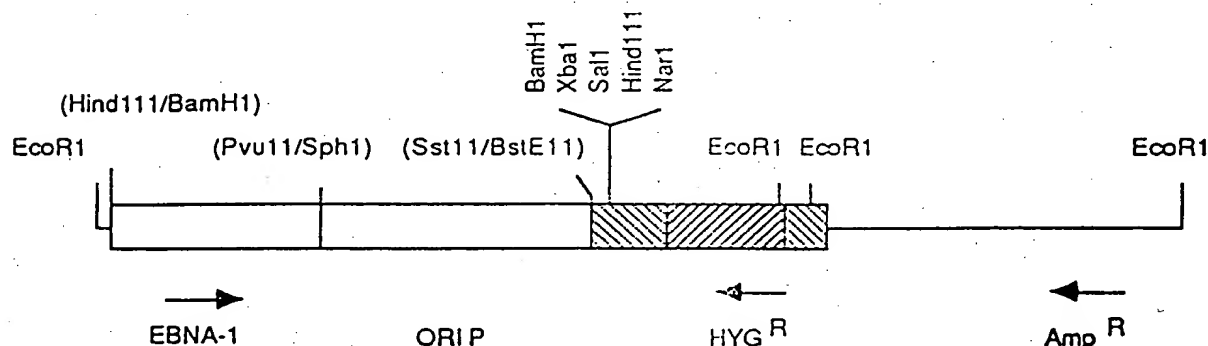
21. The use of the vector of any one of claims 2 to 10 or the pair of vectors of any one of claims 12 to 17 in the manufacture of a composition for the treatment of a disease.

10 22. A transgenic animal containing cells which contain the expression vector of any one of claims 2 to 10 or the pair of vectors of any one of claims 12 to 17.

Figure 1. EBV-Based Self-Replicating Expression Vector

p220.2

is a 8952 bp plasmid which encodes for EBNA-1, OriP and Hygromycin resistance. It replicates as plasmid in 143 and HeLa cells. EBNA-1 in this construct is driven off an unknown promoter located in the pBR322 sequences. DNA inserted upstream of EBNA-1 appears to eliminate expression of EBNA-1.



bp

1-35 — pBR322

36-2646 EBV EBNA-1 107567-110176 (Baer et. al., Nature 310:1984) Bam H1-Pvu11 fragment. Bam H1 site was blunt-end ligated to the Hind111 site.

2647-4826 EBV OriP 7333-9516 Sph1- Sst11 sites blunt-end ligated to the BstE11 site. (Sugden et.al., MCB 5: 410, 1985)

4827-5460 HSV TK regulatory region (McKnight, S.L., Nucleic Acids Res. 8, 5949, 1980.)
 6488-6747 Pvu11 fragment ligated into the poisonless pBR322 at Nae 1 site. These sites lost in cloning.

5461-6487 HPH gene (Gritz and Davies, Gene 25:179, 1983) Ban H1 fragment blunt end ligated into the Sma1 and Bgl11 sites in HSV TK sequences.

6748-8952 — pBR322 poisonless vector (deletion of 1.1 kb in pBR322) confers ampicillin resistance. (Lusky & Botchan, Nature 293:79,1981)

The polylinker from pUC 12 (Sma1-Hae111 fragment) is inserted into a Nar1 site within the HSV TK sequences. The Pst1 site in the polylinker is not unique.

(/) denotes "blunt-end ligations", these sites were not regenerated in cloning.

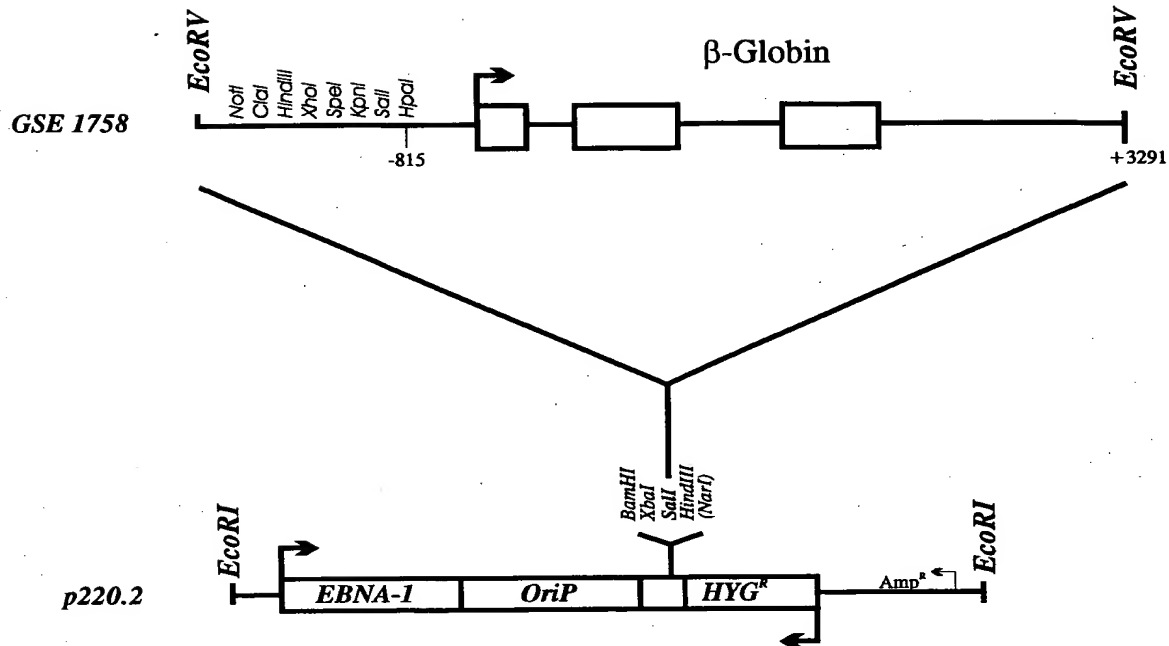


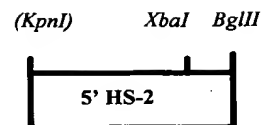
Figure 2: Reporter gene construct.

The β -globin gene extending from a 5' *HpaI* site at -815bp to an *EcoRV* site 1685bp passed the poly(A)-addition site in the plasmid *GSE1758* (Talbot et al., 1990) was removed as a 4.1kb *EcoRV* fragment and inserted into a blunted *Sall* site in the polylinker of *p220.2* (Figure 1). Note: this cloning step brings a number of extra restriction enzyme sites (including a unique *Sall* site) 5' of the β -globin gene.

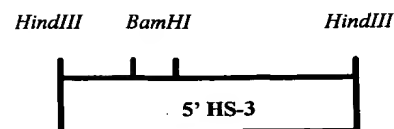
Reference:

Talbot, D., Philipsen, S., Fraser, P. and Grosveld, F. (1990) EMBO J. 9: 2169-2178.

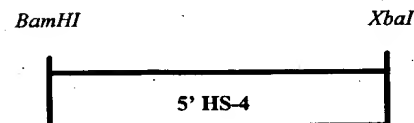
5' HS-2 - 1.5kb KpnI-BglII
blunted fragment



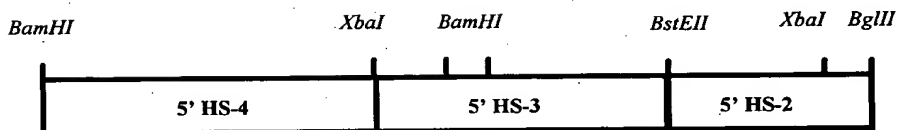
5' HS-3 - 1.9kb HindIII
fragment



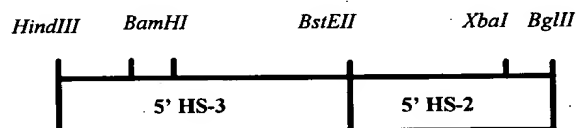
5' HS-4 - 2.1kb BamHI-XbaI
fragment



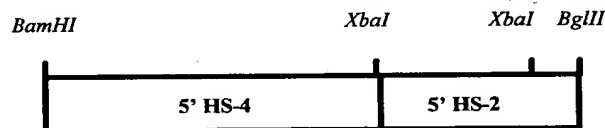
5' HS-4-3-2
5.5kb construct



5' HS-3-2
3.4kb construct



5' HS-4-3
4kb construct



5' HS-4-2
3.6kb construct

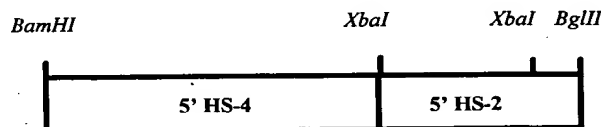


Figure 3: β -Globin LCR hypersensitive site constructs

Multiple hypersensitive site constructs retained the site order found in the wild type β -globin locus. *Sall* linkers were added to both the 5' and 3' ends allowing the DNA to be cloned into the unique *Sall* site in the β -globin-p220.2 reporter vector (Figure 2).

S1 analysis of K562 cells containing human β -globin on an EBV based vector

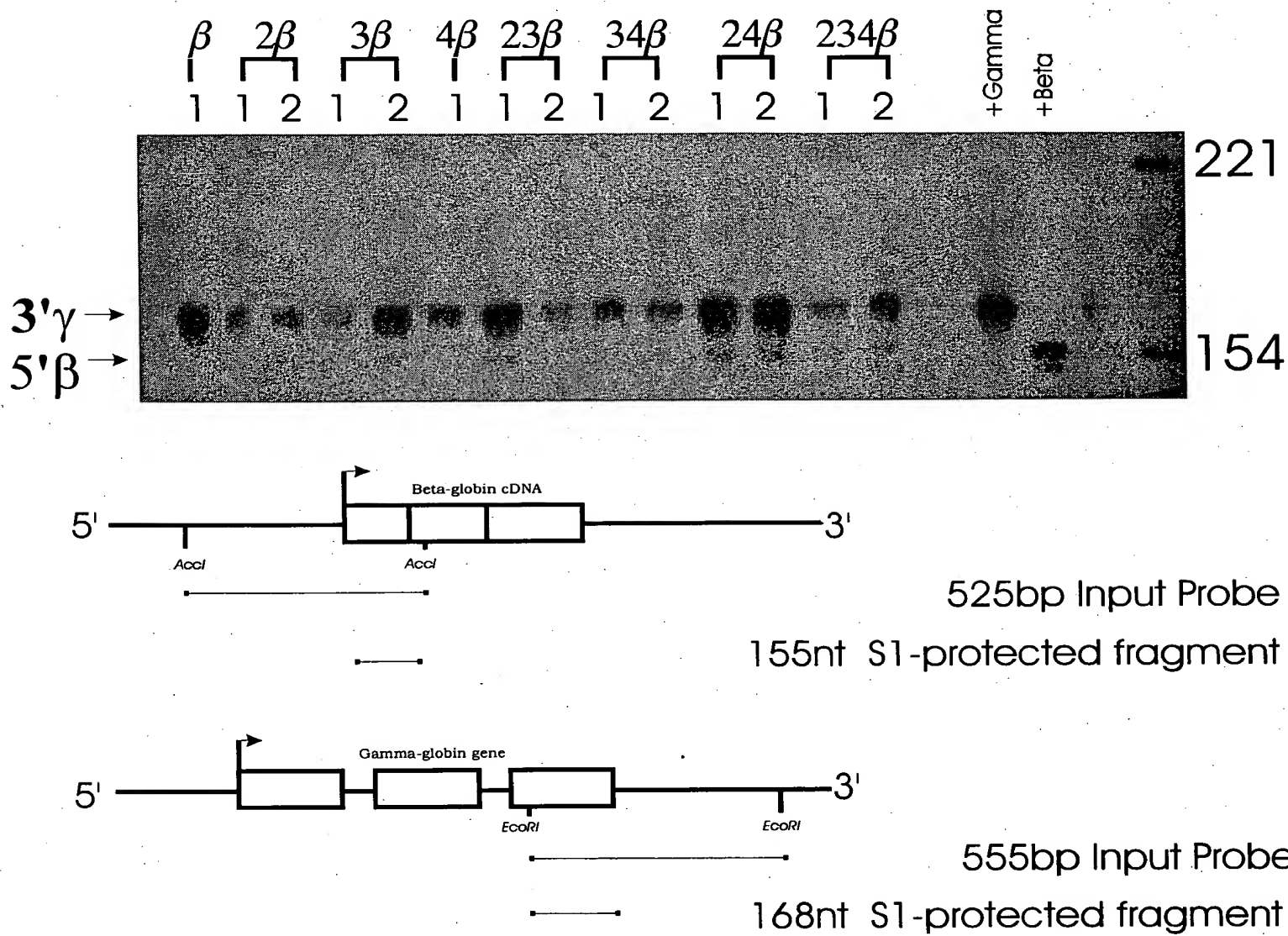


Figure 4: Numbers represent β -globin locus control region DNaseI hypersensitive site combination used.

